1-(1*H*-PYRROL-2-YL)-1,2-PROPANEDIONE IS A CRUCIAL PHEROMONE COMPONENT OF THE RARE NORTH AMERICAN CERAMBYCID BEETLE *DRYOBIUS SEXNOTATUS*

BY

NATALIE M. DIESEL

THESIS

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Master's Committee:

Professor Lawrence M. Hanks, Chair, Director of Research
Professor Andrew Suarez
Professor Brian Allan

ABSTRACT

The compound 1-(1*H*-pyrrol-2-yl)-1,2-propanedione was recently found to be an important pheromone component of several Asian species of longhorned beetles in the subfamily Cerambycinae. Here, we report the first confirmed identification of this pyrrole as a pheromone component of a cerambycine species native to North America, the rare *Dryobius sexnotatus* Linsley. Headspace volatiles from males contained (*R*)-3-hydroxyhexan-2-one and the pyrrole (ratio 1:0.13), neither of which were detected in samples from females. A field bioassay confirmed that adults of both sexes were attracted only by the binary blend of racemic 3-ketol with the pyrrole, and not by either compound alone. Adults of another cerambycine, *Xylotrechus colonus* (F.), were attracted by the ketol, consistent with its being the primary component of the pheromone of this species, and attraction was not influenced by the presence of the pyrrole. This study attests to the effectiveness of pheromone-baited traps in detecting rarely encountered species of cerambycids. It also provides further evidence that the pyrrole represents another conserved pheromone motif within the Cerambycinae.

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INTRODUCTION

Research over the last decade has provided increasing evidence that adults of woodboring beetles of the family Cerambycidae produce volatile pheromones, and that traps baited with synthesized pheromones are effective tools for monitoring populations and detecting rare species (reviewed by Millar and Hanks 2017). Many species in the two largest subfamilies, the Cerambycinae and Lamiinae, are known to use aggregation-sex pheromones (*sensu* Cardé 2014), produced by males, but attractive to both sexes. The chemical structures of some of these pheromones are highly conserved, with closely-related species (i.e., congeners), and even more distantly-related species having pheromones of identical composition, or at least sharing the same structural motif (Hanks and Millar 2016). Structural motifs that are common among cerambycines include 3-hydroxyalkan-2-ones and the related 2,3-alkanediols, whereas motifs that are common among lamiines include hydroxyethers and structures based on the sesquiterpene degradation product geranylacetone (Millar and Hanks 2017). These chemical motifs also are conserved across continents, being shared among species of cerambycids that are native to North and South America, Europe, Asia, and Australia (e.g., Fonseca et al. 2010; Hayes et al. 2016; Imrei et al. 2013; Meier et al. 2016; Pajares et al. 2013; Sweeney et al. 2014; Wickham et al. 2014).

Conversely, it is also becoming clear that species of cerambycines produce a wide variety of other pheromone components, many of which defy ready classification into related structural groups. Examples of the structural diversity of compounds which recently have been identified as pheromone components of cerambycines include (*Z*)-3-decenyl (*E*)-2-hexenoate (Ray et al.

2009), (6*E*,8*Z*)-6,8-pentadecadienal (Silva et al. 2016a), 10-methyldodecanal (Silva et al. 2016b), and 3-methylthiopropan-1-ol (Silva et al. 2017).

The compound 1-(1*H*-pyrrol-2-yl)-1,2-propanedione has recently been reported as a pheromone, or likely pheromone component of several cerambycine species. The structure was first identified from headspace volatiles of two congeners that are native to western North America, *Callidium antennatum hesperum* Casey and *C. pseudotsugae* Fisher (Hanks and Millar 2016). During field screening trials of possible pheromone components in Japan (Zou et al. 2016), it was discovered that the blend of the pyrrole and 3-hydroxyhexan-2-one attracted both sexes of *Callidiellum rufipenne* (Motschulsky), an Asian species which infests cupressaceous plants. This species colonized the northeastern United States in the 1990s, and has since spread to several states (Maier 2007). Screening trials in China subsequently revealed that the same blend attracted adults of the congener *C. villosulum* (Fairemaire) as well as adults of *Xylotrechus buqueti* (Castelnau & Gory), whereas the pyrrole alone attracted adults of *Allotreus asiaticus* (Schwarzer) and *Semanotus bifasciatus* Motschulsky (Wickham et al. 2016). In total, these species represent three tribes of the Cerambycinae, the Callidiini (*Callidium, Callidiellum, Semanotus*), Clytini (*Xylotrechus*), and Phoracanthini (*Allotreus*), suggesting that the pyrrole may be broadly shared among related taxa from several continents.

Here, we report on the first confirmed identification of 1-(1*H*-pyrrol-2-yl)-1,2-propanedione as a pheromone component of a species native to North America. The pyrrole, in combination with (*R*)-3-hydroxyhexan-2-one, comprises the male-produced pheromone of *Dryobius sexnotatus* Linsley. This species is the only member of its tribe, the Dryobiini, and is native to the eastern United States, but rarely collected (for biology, see Lingafelter 2007; Linsley 1964; Perry et al. 1974). The confirmed identification of the pyrrole compound from *D.*

sexnotatus provides further evidence that that pyrrole is indeed a conserved cerambycine pheromone motif, and also provides a reliable method for monitoring this supposedly rare species.

METHODS

Sources of Synthetic Pheromones Racemic 3-hydroxyhexan-2-one (henceforth "3-ketol") was purchased from Bedoukian Research (Danbury, CT, USA), and 1-(1*H*-pyrrol-2-yl)-1,2-propanedione ("pyrrole") was synthesized as described in Zou et al. (2016).

Collection and Analysis of Beetle-Produced Compounds The pheromone of *D. sexnotatus* was identified by collecting headspace volatiles from adult beetles on an adsorbent. The first beetle to be aerated was an adult male collected by hand on 17 June 2015 in southern Illinois (Pope County; 37.415 lat., -88.667 long.). Subsequent aerations were conducted on one male and one female that were collected during July 2016 (after the field bioassay of synthesized pheromone; see below) at a privately-owned woodlot in southwestern Illinois (Randolph County; 37.955 lat., -89.834 long.; 32 ha). The latter site was forested primarily with oaks (*Quercus* species) and hickories (*Carya* species), but also included sugar maple, *Acer saccharum* Marshall, the primary larval host of *D. sexnotatus.* In addition to sugar maple, larvae of *D. sexnotatus* also are known to develop in dead and dying elms (*Ulmus* species), ash (*Fraxinus* species), beech (*Fagus* species), and American basswood (*Tilia americana* L.) The brightly colored adults of *D. sexnotatus* are diurnal and fly primarily during June through July.

Beetles were captured with black cross-vane panel traps (corrugated plastic; Alpha Scents, Inc., West Linn, OR) that were coated with Fluon® PTFE dispersion (10% aqueous dilution; Northern Products, Woonsocket, RI, USA) to improve capture efficiency (see Graham et al. 2010). Traps were modified to capture beetles alive by replacing trap basins with 2-l plastic jars that had the bottoms cut out and replaced with aluminum screen. Traps were suspended

(~1.5 m high) from inverted L-shaped frames of polyvinylchloride irrigation pipe that were mounted on 1-m long sections of steel reinforcing bar driven partway into the ground. Pheromone emitters were polyethylene sachets (clear press-seal bag, Bagette model 14770, 5.1 × 7.6 cm, 0.05 mm thick, Cousin Corp., Largo, FL, USA) that contained a cotton roll (1 × 4 cm) to stabilize release rates. Lures were formulated to contain 50 mg of 3-ketol (i.e., 25 mg of each enantiomer), 25 mg of the achiral pyrrole, or the blend of the two, dissolved in 1 ml of isopropanol. Control lures contained 1 ml of isopropanol.

Adults of *D. sexnotatus* were sexed based on antennal length (males have longer antennae; Linsley 1964). The two males and one female that were captured alive for aeration were housed separately in aluminum screen cages under laboratory conditions (~12:12 h L:D, ~20 °C) and provided 10% sucrose solution (glass vial with a cotton wick) as nourishment. Beetles were acclimated to laboratory conditions for at least 24 h prior to aeration, and allowed at least 24 h to recuperate between bouts of aeration. Beetles were aerated individually in glass Mason-style canning jars (~0.5 l) that were sealed with a Teflon® gasket, for a total of four aerations of the males and one of the female. Aeration chambers were adjacent to closed exterior windows (natural photoperiod, ~14:10 h L:D). Charcoal-purified air was drawn through the apparatus at ~1 l/min for a period of ~24 - 48 h, and headspace volatiles were trapped with a glass collection tube containing the adsorbent HayeSep® Q (150 mg, Sigma-Aldrich, St. Louis, MO, USA) held between plugs of glass wool. Aerations of empty chambers were run simultaneously as a control to check for system contaminants. Volatile compounds were recovered from collectors by eluting with 1.5 ml of dichloromethane.

Aeration extracts were analyzed by coupled gas chromatography-mass spectrometry (GC/MS) with an Agilent 7890B gas chromatograph (Agilent Technologies, Santa Clara, CA,

USA) fitted with a DB-5ms column (30 m × 0.25 mm i.d., 0.25 μm film; Agilent J&W Columns, Agilent Technologies) and coupled to an Agilent 5977A mass selective detector. Injections were made in splitless mode with an injector temperature of 250 °C, and an oven temperature program of 30 °C for 1 min, increased 10 °C/min to 250 °C, hold 5 min. Sex-specific peaks were identified by comparing spectra and retention times with those of authentic standards.

The absolute configuration of the 3-ketone enantiomer was determined with an HP 5890 GC fitted with a Cyclodex-B column (30 m × 0.25 mm i.d., 0.25 μm film; Agilent Technologies). Injector temperature was set at 110 °C to minimize isomerization of the 3-ketone (Millar et al. 2009), with an oven temperature program of 50 °C for 1 min, increased 2.5 °C/min to 150 °C, hold 5 min (retention time of 3*R*-ketone: 18.55 min).

Field Bioassay of Synthesized Pheromones A field experiment was conducted to test for attraction of the adult beetles to racemic 3-ketol, pyrrole, and the binary blend of the two (see Results). The experiment was conducted at the study site in Randolph County, Illinois, mentioned above. Traps and lures of the designs described above were used, with the exception that trap funnels were fitted with plastic buckets that were partially filled with saturated aqueous NaCl solution to kill and preserve captured beetles. A linear transect of four traps, separated by ~10 m, was deployed on 27 May 2016 with four treatments: 1) 3-ketol, 2) pyrrole, 3) 3-ketol + pyrrole, and 4) solvent control. Traps were checked for beetles every ~3 d, at which time treatments were shifted one position along the transect to control for positional effects. The experiment was ended on 16 June 2016, for a total sample period of 18 d (i.e., seven replicates defined by collection date).

Representative specimens of species caught are available from the laboratory collection of LMH, and voucher specimens have been deposited at the Illinois Natural History Survey, Champaign, IL.

Statistics The nonparametric Friedman's Test (PROC FREQ, option CMH; SAS Institute 2011) was used to test differences between treatment means because data violated homoscedasticity assumptions of ANOVA (Sokal and Rohlf 1995). Two replicates that contained no specimens of *D. sexnotatus* were dropped from the analysis. The nonparametric Dunn-Nemenyi multiple comparison test, which limits Type I errors to acceptable levels (Elliot and Hynan 2011; Zar 2010), was used to compare pairs of treatment means.

RESULTS

Collection and Analysis of Beetle-Produced Compounds Analyses of all four extracts of headspace volatiles from males of *D. sexnotatus* revealed two peaks that were not detected in the aeration extract obtained from the female, nor from control aerations. In order of elution, the first peak was identified as 3-hydroxyhexan-2-one (retention time 6.40 min) and the second peak as the pyrrole (11.30 min; ratio 1:0.13) by comparison of retention times and mass spectra with those of authentic standards. Analysis of the extracts on a chiral stationary phase Cyclodex-B column revealed that the absolute configuration of the ketol was *R*.

Field Bioassay of Synthesized Pheromone Two species of cerambycids were captured during the field bioassay in numbers sufficient for statistical analysis: the target species *D. sexnotatus* and another cerambycine native to Illinois, *Xylotrechus colonus* (F.). Traps captured 30 adults of *D. sexnotatus* (~2:1 M:F), of which 29 beetles (97%) were in traps baited with the blend of 3-ketol + pyrrole. The mean number of beetles captured in the blend was significantly greater than that of the control treatment and the individual compounds alone (Fig. 1A; Friedman's $Q_{3,20}$ = 15.9, P = 0.0012; Dunn-Nemenyi multiple comparison test, $P < 0.05$). Adults of *X. colonus* were caught in significant numbers only by traps baited with 3-ketol alone or blended with the pyrrole (Fig. 1B; total of 57 beetles of both sexes; means significantly different; $Q_{3,28}$ = 14.2, P = 0.0026, Dunn-Nemenyi multiple comparison test, $P < 0.05$).

DISCUSSION

Attraction of adults of *D. sexnotatus* only to the blend of 3-ketol and the pyrrole confirmed that both were essential components of the pheromone. The blend of these two compounds also proved to be critical synergists for the cerambycines *C. rufipenne*, *C. villosulum*, and *X. buqueti* during earlier field trials in China and Japan (Wickham et al. 2016; Zou et al. 2016). The same field trials also revealed that adults of *A. asiaticus* and *S. bifasciatus* were attracted to the pyrrole alone, suggesting that males of those species do not produce the ketol as a pheromone component. 3-Ketol had no effect on attraction of *A. asiaticus* to the pyrrole, but apparently inhibited attraction of *S. bifasciatus*. Such differences among sympatric species of cerambycids in the synergistic and inhibitory effects of various pheromone components may have evolved to avert deleterious interspecific attraction (Millar and Hanks 2017).

(*R*)-3-Hydroxyhexan-2-one is the most common cerambycid pheromone component identified to date, being the primary or sole component of confirmed pheromones for 13 species of cerambycines, and a likely pheromone component for an additional ~50 species of cerambycines worldwide (Hanks and Millar 2016). Whereas this overlap in pheromone chemistry might seem to present opportunities for cross-attraction among species, there are several possible mechanisms by which they may remain segregated, including differences in seasonal phenology, diel activity period, or minor pheromone components which synergize attraction of conspecifics and/or antagonize attraction of heterospecifics (Millar and Hanks 2017). Mitchell et al. (2015) showed how such mechanisms serve to minimize cross attraction among eleven species of cerambycids that are native to the eastern United States, and that likely overlap in distribution with *D. sexnotatus*. Adults of *D. sexnotatus* would not be attracted by the

pheromones of these other species because they lack the critical pyrrole, but the possibility remains that the 3-ketol produced by male *D. sexnotatus* could attract other species. *Xylotrechus colonus* is among the few of these species that flies at the same time of year as *D. sexnotatus* (midsummer; see Hanks et al. 2014, Mitchell et al. 2015), and its pheromone is composed of (*R*)- and (*S*)-3-hydroxyhexan-2-one and 2,3-hexanediols (Lacey et al. 2009). In the present study, adults of *X. colonus* were attracted to 3-ketol and not influenced by the pyrrole, as was observed in an earlier study (Zou et al. 2016), suggesting that they could be attracted by the pheromone blend of *D. sexnotatus*. However, the two species are segregated by diel activity period, with *X. colonus* being crepuscular (adults flying between 8:00 and 10:00 pm; unpub. data.), whereas adults of *D. sexnotatus* fly during early afternoon (Perry et al. 1974).

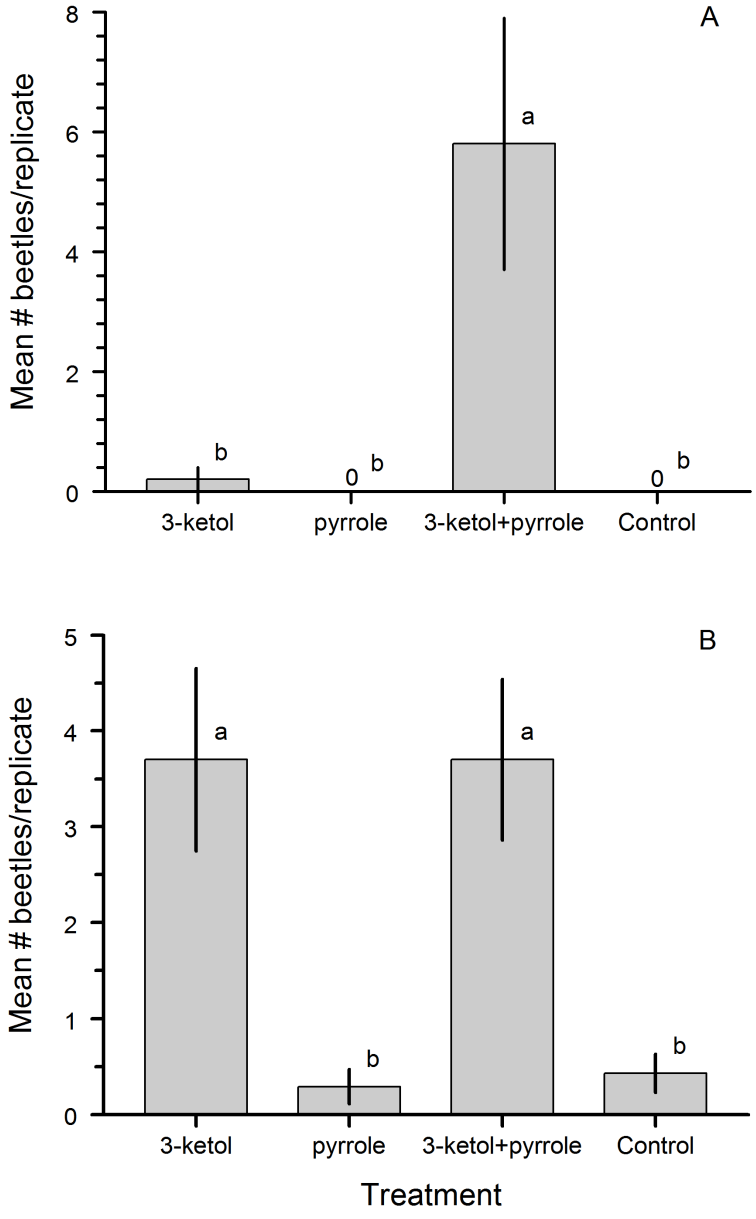
Capture of 30 adults of *D. sexnotatus* over an 18-d period, during a bioassay that included at any time only one trap baited with the attractant binary blend, is further evidence of the effectiveness of pheromone-baited traps in detecting rarely-encountered cerambycids, and in delineating their geographical distributions (Millar and Hanks 2017). This species is believed to be broadly distributed in the eastern United States (Linsley 1964, Lingafelter 2007), but our trapping surveys suggest that it is not present in some areas of Illinois. For example, during the last two years, not a single specimen was captured by sentinel traps baited with the 3-ketol + pyrrole blend in four wooded areas of east-central Illinois (L. M. Hanks, unpub. data). In fact, only two specimens of *D. sexnotatus* have been trapped during our previous ten years of intensive field trapping in these areas (Hanks et al. 2014), and both were collected at our western-most study site, Funk's Grove Nature Preserve in McLean County (40.363 lat., -89.115 long.). *Dryobius sexnotatus* may have been favored at that site by on-going successional replacement of oaks and hickories by sugar maple (McFall and Karnes 1995), which is reported

to be the most common host of the larvae (Perry et al. 1974). Our findings therefore suggest that *D. sexnotatus* is patchily distributed within its reported geographical range, but may be locally abundant where suitable host plants are abundant.

Use of the pyrrole as a pheromone component by *D. sexnotatus,* as well as reports that it is a likely pheromone component of cerambycine species of several genera that are native to North America and Asia, supports the notion that the pyrrole represents another conserved pheromone motif within the subfamily. Nevertheless, the pyrrole was a critical synergist, and not itself an attractant, only for *D. sexnotatus* in the present study. Moreover, the blend of pyrrole and 3-ketole attracted no species during season-long trapping studies conducted in east-central Illinois (see above), where there are at least 114 native cerambycid species (Hanks et al. 2014). These findings suggest that the pyrrole is not as broadly shared among sympatric species as are compounds such as the 3-hydroxyalkan-2-ones among species of the Cerambycinae and 2-(undecyloxy)ethanol among species of the Lamiinae (Hanks and Millar 2016). Thus, generic blends of common components of cerambycids, which are intended to attract a diversity of species (Hanks et al. 2012), would be ineffective in monitoring populations of *D. sexnotatus*. The same is true for other cerambycine species whose pheromone chemistry is apparently unique and species specific (e.g., Ray et al. 2009; Silva et al. 2016a,b, 2017).

FIGURES

Figure 1. Mean (± SE) number of adults of A) *Dryobius sexnotatus* and B) *Xylotrechus colonus* (sexes combined) that were caught per replicate during the field bioassay in southwestern Illinois. Chemical abbreviations: 3-ketol = racemic 3-hydroxyhexan-2-one, pyrrole = 1-(1*H*-pyrrol-2-yl)-1,2-propanedione. Means within species with different letters are significantly different (Dunn-Nemenyi multiple comparison test, $P < 0.05$).



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